INTRODUCTION

Liver diseases are common worldwide, including in Indonesia. Free radicals and reactive oxygen species (ROS) play a crucial role in developing liver diseases [1], and the liver is the first organ exposed to the damaging effects of any newly formed toxic substance. Therefore, protective armaments for the liver is a particular concern.

Bio-activation of xenobiotics such as pharmaceuticals or poisonous foods in the liver might contribute to reactive metabolic species reacting with cellular macromolecules, leading to protein malfunction, oxidative stress, lipid peroxidation, and DNA damage [2]. Despite significant improvements in pharmacological development, effective and safe hepatoprotective medicines remain in high demand [3]. Alternative therapies, such as the use of natural medicines derived from medicinal plants and their formulations, have sparked the interest of pharmaceutical companies.

Many researchers are turning to natural products because of the limited drugs used as hepatoprotectives [2]. Traditional medicine recommends a variety of herbal treatments to treat liver diseases. The majority of these treatments work as free radical scavengers, while some operate as antioxidant modulators. Thus, natural products may serve as therapeutics to cope with liver damage [1]. Among the natural products providing a wide range of antioxidants, propolis has been the subject of considerable investigation [4].

Propolis is a viscous, dark-colored substance collected by honeybees from living plants. They blend it with wax and utilise it to build and remodel their nests, mostly to seal fractures in the honeycomb and to defend against dangerous microorganisms. Propolis has been used since ancient times as folk medicine [5,6]. Because of its valuable characteristics, propolis is extensively used in complementary and alternative medicine in food and beverages to improve health, and to prevent diseases such as inflammation, heart disease, diabetes and cancer [7]. It has been claimed that propolis contains remarkable biological activities, among others, antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, anticancer properties, immunoregulatory, and bone strengthening effects owing to the aromatic acids, phenolic compounds and flavonoids found in it [8-11].

In addition, as a hepatoprotective, propolis also has the most potent antioxidant activity against oxidants and free radicals when compared to the results of investigations

Hepatoprotective effect of Indonesian propolis from Apis mellifera in carbon tetrachloride (CCl₄) induced liver injury in mice

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ABSTRACT
Propolis has been reported to have a broad spectrum of biological activities. The objective of this study was to investigate the hepatoprotective effect of propolis on liver injury induced by carbon tetrachloride (CCl₄). Twenty-five adult mice were randomly divided into five groups of five. The normal group received distilled water; positive control group was given CCl₄ at 2.8 mL/kg BW orally, other groups were given the same dose of CCl₄, followed by oral propolis at 25, 50, and 100 mg/kg BW, respectively, for 14 days. On the 15th day, the mice were sacrificed for the measurements of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, and albumin, as well as examination of liver histology. The data were analyzed using a one-way ANOVA and, subsequently, by Duncan's Multiple Range Test. The results demonstrated that propolis had a hepatoprotective effect as shown by significant improvement of the biochemical parameters (p <0.05), which was confirmed by the liver histological analytical results. The most potent hepatoprotective effect was found after the dose of 100 mg/kg BW.

Keywords: hepatoprotective, propolis, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, liver histology.
involving other bee products [12,13]. Its extract has been found to contain phenolic acids and caffeic acid phenethyl ester (CAPE), that could act as hepatoprotectors [14,15]. It has been suggested that the biological activities of propolis may be due to the presence of a large number of terpenoid and flavonoids [16]. The terpenoid content in propolis has antibacterial activity, anti-inflammatory activity and immuno-modulatory effect. Moreover, propolis has demonstrated activity in decreasing nuclear factor-KB (NF-KB) stimulation in the inflammatory cascade. Research suggests that the content of flavonoids can reduce the destructive effects of free radicals by inhibiting lipid peroxide through activation of peroxidase to haemoglobin, an endogenous antioxidant [17]. Propolis has also been found to capture hydroxyl and superoxide radicals and neutralize free radicals, hence can maintain the structural integrity of cells/tissues and membrane lipids against undesirable reactions [18]. This current study aims to determine the hepatoprotective effect of propolis on liver damage caused by CCl₄, with ALT, AST, ALP, Albumin and Total Protein as parameters.

MATERIALS AND METHODS

Materials

Carbon tetrachloride (Merck, Germany), sesame oil (PT. ABC, Indonesia), formaldehyde (Sigma Aldrich, Singapore), sodium carboxymethylcellulose 0.5% (PT. Brataco Indonesia), monosodium and disodium phosphate (PT. Brataco Indonesia), alcohol 96% (Sigma Aldrich, Singapore), sodium chloride 0.9% for injection (Otsuka, Indonesia), aqua injection (Otsuka, Indonesia). Propolis from *Apis mellifera* subsp. *mellifera* was purchased from local bee farmers in Solok, West Sumatra, Indonesia.

Extraction of propolis

The Propolis (1 kg) was cut into small pieces and extracted with 96% ethanol (1:10, w/v) at room temperature. The main constituents of propolis were previously reported by Trusheva, et al. [19].

Animals and experimental design

All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive of 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by The Research Ethics Committee, Faculty of Medicine, Universitas Andalas (No. 177/KEP/FK/2020). Twenty-five BALB/c mice aged 2-3 months weighing 20-30 grams were used in this research. All animals were acclimatized to laboratory condition for 1 week before the study. Male BALB/c mice weighing 20-30 g were used in this research. The animals were divided into five groups. The normal control group was treated with distilled water and received daily oral administration of 1 ml/kgBW of propolis solution for 7 days. The experimental groups were treated with the same volume of propolis solution. The normal group received distilled water. The positive control group received CCl₄, with ALT, AST, ALP, Albumin and Total Protein as parameters.

RESULTS

Effect of Propolis on Serum Biochemical Markers Levels

This study found that the addition of CCl₄ significantly increases the activities of ALT, AST, and ALP when compared to the normal group. In contrast, propolis supplementation resulted in significantly lower ALT, AST and ALP levels (p <0.05) in the animal group. Propolis dose 100 mg/kg BW gave the best activity at ALT and ALP levels (32.3±5.04 U/L; 68.4±7.44 U/L), compared to the normal control group (17.14±8.83 U/L; 133.55±12.37 U/L). Moreover, propolis dose 50 and 100 mg/kg BW gave the best activity on AST level, which gives an equal result.
(51.06±6.01 U/L), as compared to the normal control group (24.90±8.44 U/L) – as seen in Figure 1. The treatment of propolis extract (PE) at 50 mg/kg BW and 100 mg/kg BW recovered the impaired liver functions induced via CCl₄ damage.

**Effect of Propolis on Albumin and Total Protein Level**

Table 1 presents the results of albumin and total protein measurements. The levels of albumin (2.86 g/L) and total protein (4.81 g/L) were significantly lower in the CCl₄ group compared to the normal control group (p <0.05), indicating liver injury. Propolis supplementation significantly improved these condition (p<0.05), bringing them back to normal in a dose-dependent manner. PE 100 provided better results, with comparable levels to the control group.

**Histology Analysis**

Histology results in the CCl₄ control group showed degeneration of parenchyma, hydropic degenerative and necrosis. In the hepatocyte cell degeneration, we can see the granular in the cytoplasm, as well as an enlarged cell size because of a swelling cell. Figure 2 illustrates the liver histopathology in a qualitative manner, providing the liver description of this study. In the positive control group, the mean histopathology score increased during CCl₄ induction to as high as 3.74±0.12. This is significantly different compared to normal controls (p<0.05), as seen in Table 2. The histopathology score of PE 100 demonstrated the greatest hepatoprotective effect (2.92±0.13), and is statistically different to other propolis doses.

**DISCUSSION**

This study investigated the effects of CCL4 administration on liver cells and the effect of propolis administration on preventing hepatocyte damage. Hepatic function marker such as the enzymes ALT, AST and ALP characterize hepatocyte damage. When liver cells are injured, these enzymes are released from cells into the circulation and generate significantly elevated serum levels[19]. Our findings showed that CCl₄ administration causes severe acute liver injury in mice. This finding validates the previous study’s finding that CCl₄ is a preferred chemical agent for inducing hepatic cell damage in animals.[20,25] Hepatic damage induction with CCl₄ is potentially reversible, and it precisely models actual liver cirrhosis by producing free radicals and inducing pro-inflammatory and pro-fibrotic cytokines. In the liver, CCl₄ is metabolized to CCl₃. Once exposed to O₂, it transforms from CCl₃ to CCl₃O₂- CC₃O₂ and CCl₃O₂ – which are highly reactive lipid and protein binders. All of the structural changes generated by free radicals will result in a decrease in protein production. Damage to the cytoplasmic membrane lipids will eventually result in a rise in hepatic serum indicators such as ALT, AST and ALP [26,27].

CCl₄ exposure caused severe liver damage in the current study, as seen by higher serum ALT, AST, and ALP levels. These cytoplasmic marker are released into the blood following cellular injury [28]. This research revealed the effects of a combination of hepatic damage, hepatocellular (predominantly first ALT elevation) and cholestatic (initial ALP rise). AST acts as a catalyzer in converting alanine to pyruvate and glutamate. AST thus could be a better biomarker of liver damage. When liver cells are injured, these enzymes are released from cells into the circulation and generate significant elevated serum levels [19]. Our findings showed that CCl₄ administration causes severe acute liver injury in mice. In these conditions, there was less of an elevation in ALT level. Meanwhile, serum ALP is associated with hepatic cell activity. An elevation in serum ALP is caused by increased synthesis due to increasing biliary pressure [27].

These present study findings demonstrated a decline in the levels of serum marker enzymes following PE administration. In this condition, the free radicals produced during CCl₄ biotransformation must be either eliminated or suppressed to reduce liver injury. Mice with CCl₄ – induced liver damage treated with PE at 25, 50 and 100 mg/kg BW were able to reduce AST, ALT and ALP. In this study, the ALT and AST values in the test group were still higher than in the normal group. However, these were much lower than that in the positive control group. This happens because the hepatocyte cell repairing process is still going on and takes a long time. These conditions indicate the hepatoprotective effect

**Table 1.** The effect of propolis extract (PE) on albumin and total protein level in CCl₄-induced mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Albumin (g/L)</th>
<th>Total Protein (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.48±0.84</td>
<td>6.77±0.72</td>
</tr>
<tr>
<td>Positive control</td>
<td>2.86±1.09⁴</td>
<td>4.81±2.31⁴</td>
</tr>
<tr>
<td>PE 25</td>
<td>3.31±0.71⁴</td>
<td>5.94±2.17⁴</td>
</tr>
<tr>
<td>PE 50</td>
<td>3.58±0.63⁴</td>
<td>6.46±1.07⁴</td>
</tr>
<tr>
<td>PE 100</td>
<td>4.43±0.61⁴</td>
<td>7.22±1.45⁴</td>
</tr>
</tbody>
</table>

Data are the mean ± SE of five mice, a=significant difference compared to normal group (p<0.05); b=significant difference compared to positive group (p<0.05)

**Table 2.** The effect of propolis on liver histopathology score in CCl₄-induced mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver histopathology score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.01±0.02</td>
</tr>
<tr>
<td>Positive control</td>
<td>3.74±0.12⁴</td>
</tr>
<tr>
<td>PE 25</td>
<td>3.04±0.14⁴</td>
</tr>
<tr>
<td>PE 100</td>
<td>2.92±0.13³</td>
</tr>
</tbody>
</table>

Data are the mean ± SE of five mice, a=significant difference compared to normal group (p<0.05); b=significant difference compared to positive group (p<0.05)
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The arrow pointed to S: Sinusoids, and VS: Central vein

Figure 2. Histology of liver mice cells in CCl₄-induced mice in normal group (A), CCl₄ control group (B), Normal group (C), Propolis 25 group (D) Propolis 50 (E) Propolis 100 group. H & E was used to stain 6-mm slices of liver at a magnification of 40x. The circle represented normal cells with intact nuclei and regular sinusoids.

of propolis. This finding was backed by Nakamura et al. [29] The optimal effect in reducing ALT, AST and ALP shown in dose PE100, as seen in Table 1.

Caffeic Acid Phenethyl Ester (CAPE) is a natural chemical substance in propolis that acts as an antioxidant [30,31]. Changes in ALP, ALT and AST enzyme in the animal group given propolis is associated with the propolis compounds that act as antioxidants. Antioxidants are substances that neutralise a free radical [14]. Sharma and Shukla supported this finding (2011), by reporting that the effect of antioxidant flavonoids can improve the process of regeneration [20,29]. CAPE has 4-6 times antioxidant activity more potent against oxidants and free radicals than vitamin C and N-acetyl-cysteine (NAC) [13]. Propolis can catch radical hydroxyl and superoxide, then neutralize free radicals, hence protecting cells by maintaining their structural integrity, and protecting lipid membranes against undesirable reactions [15].

The largest plasma protein in the bloodstream is albumin, solely made in the liver. It participates in the scavenging of oxygen free radicals. It is also crucial in distributing various compounds in the circulation linked to albumin, including medicines, lipids, hormones and toxins. As the results of our study show a rise in total protein content, this outcome might be regarded as amelioration in the degree of cellular dysfunction in liver disorders [32]. The presence of a high albumin level in the blood indicates cellular leakage and a lack of functional integrity of the cell membrane in the liver. Protein synthesis stimulation has been proposed as a contributing hepatoprotective mechanism that accelerates liver enzyme deformation and production [33].

Although ALT, AST and ALP are considered sensitive enough for indicating the presence of liver cell damage, these measurements lack specificity, so a histology study is necessary to identify microscopical liver damage [34,35]. Histology results in the normal control group (Figure 1) revealed intact central venous and nucleus cells, with no inflammation cells, and that some cells had undergone necrosis. However, this is not included in the incidence of pathology, because necrosis may also occur [29,36].

According to Trefts et al., parenchymal degeneration occurs due to failure of oxidation, leading to disturbed protein transport produced by the ribosome, which results in an accumulation of water in the cell that causes swelling. Moreover this effect induces the appearance of granules in the cytoplasm due to protein sediment [23,37]. In the group dose of 25, 50 and 100 mg/kg BW (Figure 2), there is a decrease in liver damage histology. This was characterized by fewer necrosis cells, normal sinusoid and less damage in the central vein compared to the positive control group. PE100 administration demonstrated the highest decrease in histology score. This came about because of the dose-dependent activity thus, protection of liver damage will also be higher than other doses. Overall, it was concluded that propolis, because of its antioxidant and anti-inflammatory activities, works as a potent hepatoprotective agent and protects the liver from severe CCl₄-induced damage.

CONCLUSION

This study shows that propolis has hepatoprotective effect by significantly affecting ALT, AST and ALP levels (p <0.05) and significantly restoring the albumin and total protein level to normal (p<0.05). These outcomes were confirmed by the liver histology score in the carbon tetrachloride (CCl₄) – induced mice. Propolis at the dose of 100 mg/kg BW had the highest hepatoprotective effect.

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